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# HUMAN LYMPHOCYTE PROLIFERATIVE RESPONSE TO A SPOROZOITE T CELL EPITOPE CORRELATES WITH RESISTANCE TO FALCIPARUM MALARIA[1]

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**To identify vaccine relevant T cell epitopes on the circumsporozoite (CS) protein of *Plasmodium falciparum*, the lymphocyte proliferative responses to 10 CS protein derived peptides were studied in 28 adult Kenyans, and correlated with resistance to malaria. Eight peptides, six of which were not overlapping, induced proliferation of lymphocytes from one to five volunteers, suggesting either genetic restriction of response to each of the T epitopes, or dominance of some T sites on the immunizing sporozoites. The 28 volunteers were radically cured of malaria and during the next 126 days 25 of the 28 were reinfected. Resistance to malaria was not correlated with antibodies to malaria Ag, but was significantly correlated with lymphocyte responses to CS protein residues 361-380 and 371-390. Among the 25 volunteers who became re-infected with malaria, lymphocytes from only two responded to a peptide including residues 361-380 of the *P. falciparum* CS protein, and only one to peptide 371-390. In contrast, lymphocytes from all three volunteers who did not become infected responded to peptide 361-380 ($p = 0.003$), and lymphocytes from two of the three responded to peptide 371-390 ($p = 0.023$). The significant correlation between proliferation to peptides 361-380 and 371-390 and resistance to malaria suggests that at least one epitope within these overlapping peptides is involved in a protective cellular immune response. The data support inclusion of these residues in future CS protein vaccines.**

Development of effective subunit vaccines requires identification of relevant epitopes and a method of immunization that induces an optimal immune response. The first human malaria vaccines have been designed to elicit antibodies to sporozoites, the stage of the parasite transmitted to man by mosquitoes. A repetitive epitope on the CS[3] protein of *Plasmodium falciparum* was selected as the target antigen, because antibodies to the analogous epitope on a murine malaria CS protein protect mice against infection with sporozoites (1, 2), and antibodies to the *P. falciparum* repeat region mediate *in vitro* reactions thought to indicate protective immunity (3–6). However, the subunit sporozoite vaccines tested to date have been poorly immunogenic for humans, and only two of five volunteers who received the highest doses of these vaccines were protected against sporozoite-induced malaria (7, 8). One method for improving the antibody response to the CS repeat region would be to include additional Th epitopes in a vaccine. If these were derived from the CS protein, boosting of antibodies might occur after exposure to sporozoites (9, 10), and repeated doses of vaccine might not be required after primary immunization.

T cell epitopes are also important targets for the protective cell-mediated immunity that develops after immunization with irradiated sporozoites (2, 11–13). The effector arm of such immunity apparently requires lymphocytes of the suppressor/cytotoxic phenotype and IFN-γ (12, 13). The Ag responsible for this potent protective immune response are unknown. They may include T epitopes on the CS protein, because immunization of mice with an attenuated strain of *Salmonella typhimurium* transformed with the *P. berghei* CS gene induces protection against sporozoite challenge in the absence of anti-sporozoite antibodies (14).

Most adults in malaria endemic areas have antibodies to the repeat region of the CS protein (6, 15), but few have T lymphocytes sensitized to the same epitope (16–19), suggesting the existence of other Th epitopes (17, 19). These adults develop an immune response that renders them less susceptible to malaria than are children, and some adults less susceptible than others. If this immune response is directed against sporozoites, it is probably cellular in nature, because naturally acquired antibodies to sporozoites do not prevent human malaria infection (15). In the present study we identified non-repeat region, CS protein T epitopes that may provide help for produc-

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[3] Abbreviations used in this paper: CS, circumsporozoite protein; R32LR, a protein synthesized in *Escherichia coli*, that included 32 tetrapeptides derived from the *P. falciparum* CS protein; MDP [(NANP)$_4$ NVDP]$_4$LR; peptides 361-380 and 371-390, synthetic peptide corresponding to residues 361-380 and 371-390 of the deduced amino acid sequence of the 7G8 clone of *P. falciparum*; SI, stimulation index.

tion of antibodies to the repeat region, and demonstrated that the lymphocyte proliferative response to at least one epitope correlates with resistance to malaria.

## MATERIALS AND METHODS

*Selection of volunteers.* In April 1987, volunteers who had participated in a study conducted a year earlier (15) were informed of the study. Thirty adult, male, life-long residents (mean ± SD age = 30.2 ± 8.55 yr) of Saradidi, a holoendemic malarious area of Kenya, volunteered, gave informed consent, and donated blood. One volunteer dropped out after 3 wk of the study, and serum was not available from one, leaving 28 volunteers for the final analysis.

*Ag.* To define T epitopes, we stimulated lymphocytes with R32LR (10), a purified recombinant protein [MDP[(NANP)$_{15}$NVDP]$_2$LR] derived from the central repeat region of the *P falciparum* CS protein, and nine synthetic peptides (Fig. 1), based on the deduced amino acid sequence of the 7G8, Brazilian clone of *P. falciparum* (20). The synthetic peptides all included 20 amino acids derived from the CS protein and a carboxyl-terminal cysteine residue, and were prepared and purified by R. A. Houghten (Scripps clinic and Research Foundation, La Jolla, CA) as previously described (17, 21).

*Lymphocyte proliferation.* Heparinized blood was obtained from volunteers in their village and transported to Nairobi (approximately 3 h) where lymphocyte proliferation assays were performed as previously described (7). Based on our experience (7, 10), an Ag concentration of 15 μg/ml was chosen for all assays. The response to an Ag was considered positive if the SI index (ratio of stimulated to unstimulated cells) was greater than 3 SD above the mean SI of cells from four to six American volunteers tested simultaneously.

*ELISA.* ELISA were performed as described (15) at a serum dilution of 1/100, using R32LR (Fig. 1)(10) as Ag. A serum was considered to have antibody if the absorbance was greater than 3 SD above the mean absorbance of 10 sera from residents of Washington, D.C.

*Determination of incidence.* To determine incidence of *P. falciparum* infection, on day 0 after blood was obtained, each of the 28 volunteers was treated with 3 tablets of 25 mg pyrimethamine/500 mg sulfadoxine (Fansidar, Roche Laboratories, Nutley, NJ), followed by 100 mg of doxycycline twice daily for 7 days; a regimen expected to achieve radical cure (15). Each volunteer was visited daily for the next 126 days. Malaria smears were made on days 7, 14, 21, 28, 42, 56, 70, 84, 98, 112, 126, and on any day a volunteer complained of illness. Malaria thick smears were considered negative after two microscopists read 200 oil immersion fields (×1000).

*Entomologic inoculation rate.* Indoor biting collections for determination of the man-biting rate and sporozoite rate were conducted weekly, beginning 2 wk before the first blood drawing and continuing through day 126. The entomologic inoculation rate (number of bites by anopheline mosquitoes with *P. falciparum* sporozoites in their salivary glands per individual per night) was calculated based on this data (J. C. Beier, manuscript in preparation).

## RESULTS

*Identification of T cell epitopes.* Unstimulated cells incorporated 352 ± 246 counts of [$^3$H]TdR per min (mean ± SD). At a concentration of 15 μg/ml, R32LR stimulated a proliferative response in lympocytes from two individuals (Table I), confirming our observation that the repeat region contains a human T cell epitope (7). Seven of the nine synthetic peptides, five of which are non-overlapping, also elicited a positive response, indicating that there are at least five non-repeat region, human T epitopes on the *P. falciparum* CS protein. One of these epitopes is within residues 326-345, a highly amphipathic region of the CS protein, and a region previously shown to contain a murine Th cell epitope (9). However, more individuals responded to a peptide, 361-380, from a region with a lower amphipathic score (9, 22); an observation also made in Gambia (17).

*T cell proliferation and antibodies to CS protein.* The relationship between T cell responses to CS peptides and antibody responses to repeat epitopes indicates that some of the non-repeat region T epitopes provide help for production of antibodies to the repeat region and can be exploited in the development of CS subunit vaccines. Ten of 11 (91%) individuals with a proliferative response to at least one of the peptides had antibodies to the CS repeat region (Table I) (15), compared with 10 of 17 (59%) individuals who did not respond to any peptide, and the level of antibodies among the 11 responders was significantly higher than among the 17 non-responders (0.73 ± 0.512 vs 0.43 ± 0.238, $p < 0.025$, Student's *t*-test, two-tailed). The difference was due to the antibody levels in those whose T cells responded to R32LR, and peptides 351-370 and 361-380. The mean absorbances of the two individuals who responded to R32LR (1.42 ± 0.495), and the six individuals who responded to peptides 351-370 or 361-380, but not to R32LR (0.72 ± 0.395) were significantly higher than that of the 17 individuals who did not respond to any peptide (0.43 ± 0.238) ($p < 0.025$, Student's *t*-test, two-tailed).

*Re-infection with malaria after radical cure.* Eleven of the volunteers had *P. falciparum* parasitemia on day 0 and 27 volunteers had negative malaria smears on days 7 and 14, and all on day 21. The one volunteer with parasitemia on day 14 was treated with amodiaquine and did not develop recurrent malaria during the remainder of the study. During the 126 days of the study each individual was estimated to have been bitten by more than 100 mosquitoes infected with *P. falciparum* sporozoites. Recurrent parasitemia developed between day 28 and 126 after radical cure in 25 of the 28 volunteers (Fig. 2).

*Proliferative response and resistance to malaria.* Among the 10 Ag tested, only for peptides 361-380 and 371-390 was there a correlation between the proliferation to the Ag and resistance to malaria. The proportion of responders and the level of response (by SI) to these peptides were significantly greater among the individuals who were not reinfected than among those who were reinfected (Table II).

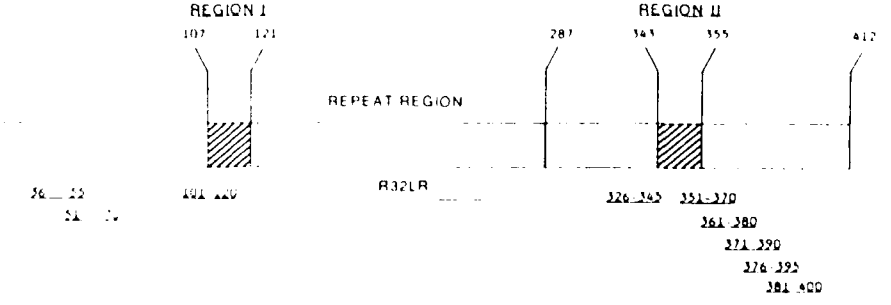


*Figure 1.* Schematic diagram of the CS protein of *P. falciparum* and the CS protein-derived peptides used to stimulate lymphocytes. The central portion of the CS protein contains 41 tandem tetrapeptide repeats (37 NANP and 4 NVDP). Region I and II refer to amino acid sequences that are relatively conserved among CS proteins from different plasmodial species (20). The nine non-repeat region synthetic peptides are designated by residue number.

TABLE I

*Lymphocyte proliferative response to CS protein Ag and level of antibodies to the CS protein repeat region in 11 individuals whose lymphocytes proliferated after stimulation with at least one peptide*

| Antibody to CS Protein[a] | Stimulation Indices to each Peptide in Positive Responders | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 36-55 | 101-120 | R32LR | 326-345 | 351-370 | 361-380 | 371-390 | 376-395 |
| 0.09 | | | | | | | 5.58 | |
| 0.34 | | | | 30.21 | | | | |
| 0.34 | | | | 6.64 | | 7.85 | | |
| 0.39[b] | | | | | | 5.85 | 6.49 | |
| 0.44 | 4.32 | 2.32 | | | | | | |
| 0.56 | | | | | 3.55 | | | |
| 0.77[b] | | | | | | 14.65 | 12.42 | 4.39 |
| 0.82 | | | | | 2.99 | | | |
| 1.07[b] | | | 5.4 | | | 15.57 | | |
| 1.42 | | | | | | 5.58 | | |
| 1.77 | | | 6 | | | | | |

[a] The absorbance (414 nm) of sera at a dilution of 1/100.
[b] These three individuals did not develop recurrent malaria during the 126-day observation period.

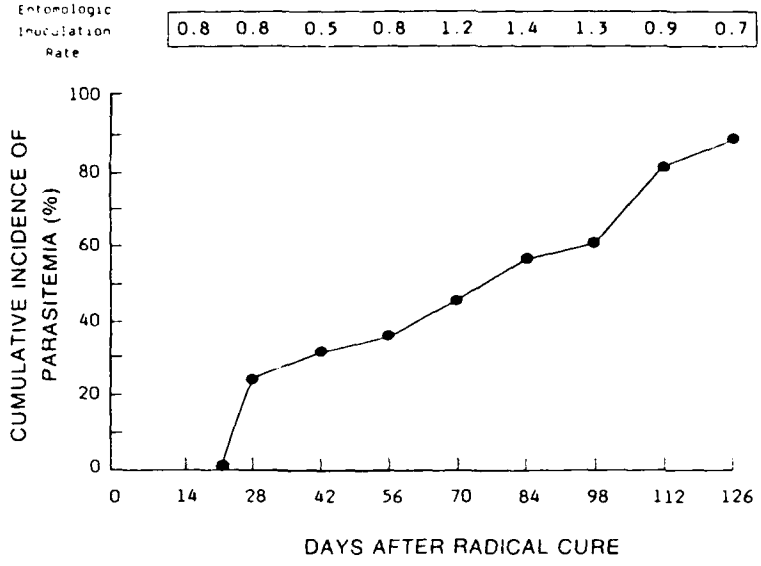


*Figure 2.* Cumulative incidence of *P. falciparum* infections after radical cure and the entomologic inoculation rate during the 126 days of the study. The values for entomologic inoculation rate represent the mean entomologic inoculation rate during the preceding 14 days.

TABLE II

*Comparison of day 0 lymphocyte proliferative responses to stimulation with peptides 361-380 and 371-390 in the 25 volunteers who did and the three who did not develop recurrent parasitemia[a]*

| Developed Recurrent parasitemia | Stimulation with Peptides 361-380 and 371-390 | | | | |
|---|---|---|---|---|---|
| | N | No. Positive | | SI | |
| | | 361-380 | 371-390 | 361-380 | 371-390 |
| Yes | 25 | 2 | 1 | 1.5 ± 1.90 | 1.3 ± 1.38 |
| No | 3 | 3 | 2 | 12.0 ± 5.37 | 6.7 ± 5.69 |

[a] The results are expressed as the number of volunteers with a positive proliferative response, and the mean ± SD SI of the entire group. The group that did not develop recurrent parasitemia had a significantly greater proportion of responders to peptides 361-380 and 371-390 ($p = 0.003$ and 0.023, respectively, Fisher's exact test, one-tailed), and a significantly greater mean SI ($p < 0.0005$, Student's *t*-test, two-tailed) than did the group that did develop recurrent parasitemia.

## DISCUSSION

These studies delineate non-repeat region T epitopes on the CS protein of *P. falciparum*, suggest that some of these sites are involved in providing help for production of antibodies to the repeat region, and for the first time identify a region of the CS protein that may be involved in a protective cellular immune response.

Most individuals failed to respond to each peptide. This was apparently not due to malaria-related immunosuppression, or to an inappropriate in vitro dose of Ag, because repeating the assays 21 days after radical cure of malaria and testing with Ag concentrations from 0.23 to 60 μg/ml did not significantly alter the results (C. F. Mason, manuscript in preparation). It may reflect genetic restriction of human response to T epitopes of the *P. falciparum* CS protein (19), a phenomenon demonstrated in mice for two regions of the *P. falciparum* CS protein (9, 23, 24), or the masking of the response to poor T epitopes on the CS protein by more dominant ones on the sporozoite, as has been shown for lysozyme (25). Despite the poor response to all the peptides, most individuals made antibodies to the repeat region. T cell help for production of antibodies to the repeats could have been provided by non-CS protein Th cell epitopes on sporozoites. However, the data suggest that a repeat region epitope and non-repeat region T cell epitopes on the CS protein, specifically those included within residues 351-380, provide T cell help for repeat antibodies. This interpretation is consistent with our finding that there are adequate Th cell sites on the *P. berghei* CS protein to completely overcome genetic restriction of response to the predominant *P. berghei* CS protein repeat.[4]

[4] Hoffman, S. L., J. A. Berzofsky, D. Isenbarger, E. Zeltzer, W. R. Majarian, M. Gross, and W. R. Ballou. Immune response gene regulation of immunity to *Plasmodium berghei* sporozoites and circumsporozoite protein vaccines: Overcoming genetic restriction with whole organism and subunit vaccines. *Submitted for publication.*

Perhaps, the most important finding was the correlation between proliferation to peptides 361-380 (IKPGSANKPKDELDYENDIEC) and 371-390 (DELDYENDIEKKICKMEKCSC) and resistance to re-infection with malaria (Table II). The three individuals who did not develop recurrent malaria and the 25 individuals who did had similar levels of antibodies to the repeat region of the CS protein, consistent with our previous findings, that in this village naturally acquired antibodies to sporozoites do not protect against malaria (15). Individuals in the two groups also had similar levels of antibodies to asexual, blood stages of *P. falciparum*. The significant difference between the non-infected and infected individuals in T cell proliferative responses to peptides 361-380 and 371-390 suggests that the proliferation reflects a protective cellullar immune response. It is intriguing to speculate that one or more epitopes included within residues 361-390 are involved directly, or indirectly via Th cells, in a $CD8^+$ cytotoxic T cell-mediated protective immune response,[5] analogous to that demonstrated in the murine malaria models (12, 13). This can only be proven by immunization and challenge studies in human volunteers. However, this hypothesis is supported by preliminary experiments with an attenuated strain of *S. typhimurium* transformed with portions of the *P. berghei* CS gene. The carboxyl-terminal portion of the *P. berghei* CS protein, containing the region analogous to *P. falciparum* residues 361-390, appears to be required for induction of protective immunity (W. R. Ballou, personal communication).

Peptides 361-380 and 371-390 include residues known to vary among strains of *P. falciparum* (26). These peptides were derived from a Brazilian strain, and the three protected individuals were exposed to more than 100 infective bites in East Africa. If the response to these peptides reflects a protective immune response, either Kenyan and Brazilian parasites are conserved at the relevant site(s) along this sequence, or the protective response associated with these antigens is not restricted by minor amino acid substitutions.

The identification of a region of the *P. falciparum* CS protein that includes at least one T cell site that may be involved in a protective cellular immune response supports including this region in future malaria vaccines. However, only 21% of the individuals in this study responded to peptides 361-380 or 371-390, whereas all volunteers immunized with large numbers of irradiated sporozoites were protected against sporozoite challenge (27–30). This suggests that there are other protective T cell sites on the sporozoite or exoerythrocytic stages of *P. falciparum*, or that experimental immunization with large numbers of sporozoites over a short period of time induces a more potent immune response than does long term natural exposure to small numbers of sporozoites. Major challenges to further vaccine development will be to determine if there are additional protective T sites, and to develop methods of immunization that induce the required T cell immunity.

[5] Since completion of this work residues 368-390 have been found to include the only cytotoxic T cell site on the *P. falciparum* CS protein in BIO BR (H-2$^b$) mice (Kumar S., L. H. Miller, I. A. Quakyi, D. B. Keister, R. A. Houghten, W. L. Maloy, B. Moss, J. A. Berzofsky, and M. F. Good. 1988. Cytotoxic T cells specific for the circumsporozoite protein of *Plasmodium falciparum*. *Nature* 334:258).

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